Development and Evaluation of Semicontinuous Slurry Microcosms to Simulate *in Situ* Biodegradation of Trichloroethylene in Contaminated Aquifers

URSULA JENAL-WANNER AND PERRY L. MCCARTY*

Department of Civil and Environmental Engineering, Stanford University, Stanford California 94305-4020

Trichloroethylene (TCE) was efficiently biodegraded *in situ* by aerobic cometabolism at the Moffett Federal Airfield test site, in the presence of phenol or toluene (Hopkins et al. *Appl. Environ. Microbiol.* **1993**, *59*, 2277; Hopkins et al. *Environ. Sci. Technol.* **1993**, *27*, 2542; Hopkins, G. D.; McCarty, P. L. *Environ. Sci. Technol.* **1995**, *29*, 1628). Using uncontaminated soil from this site as an inoculum, a semicontinuous slurry microcosm method was developed that reproduced *in situ* TCE degradation rates. The slurry method was then applied to evaluate the *in situ* TCE biodegradation potential at Edwards Air Force Base where full-scale application was contemplated. With 0.66 mg/L TCE and either 9.7 mg/L toluene or 13.4 mg/L phenol in the exchange fluid, steady state TCE removals varied between 87 and 99%, depending upon sample depth. Toluene and phenol growth yields based upon oxygen to substrate ratios were 0.77 and 0.59 g/g during the start-up phase and 0.52 and 0.36 g/g during steady state TCE removal, respectively. The TCE degradation rate constant ratio, k_c/K_c, was similar for the two substrates. Steady state TCE removals varied little in a given microcosm over 1 year of operation. In the Moffett and Edwards microcosms as well as at Moffett Federal Airfield (Fries et al. *Appl. Environ. Technol.* **1997**, *63*, 1523), toluene *o*-monooxygenase was the dominant TCE-oxidizing enzyme present.

Introduction

In situ bioremediation of chlorinated solvent contaminants offers promise as a nondisruptive technique for groundwater cleanup. Along with physicochemical, geological, and hydrological parameters, the feasibility of a given contaminated site to undergo *in situ* bioremediation is dependent on the capacity of the intrinsic microbial population to degrade the compound(s) of interest given the appropriate stimulating conditions. The biodegradation potential of a specific organic contaminant at a given site needs to be determined prior to design and construction of a treatment system, generally through laboratory studies. However, even if laboratory screening experiments suggest good biodegradation potential, a major concern remains; that is, are laboratory test conditions applicable and comparable to processes that will occur in the field?

* Corresponding author phone 650-723-4131; fax: 650-725-9474; e-mail: mccarty@ce.stanford.edu.

Over the past several years at Stanford University, several approaches to laboratory testing of the biodegradation potential of aquifer material in microcosms have been evaluated (*5*−*7*). For the most part, the microcosms used were glass columns containing aquifer material as this was felt to best simulate the natural processes that would occur in aquifers. However, column microcosms were found to have several limitations that led to the modified approach reported here. First, disturbed aquifer material when placed in laboratory columns exerts an oxygen demand much greater than experienced in the field, thus limiting the concentration of primary substrate that can be added because of oxygen limitations. Second, small columns (25 mL) were found most desirable for use because of limited aquifer material availability, the desire to test many different conditions at a time, and the necessity to operate columns under aseptic conditions (*6*). However, this limits the amount of sample available for analysis. Third, at some sites, sorption of chlorinated solvents to aquifer material is a significant phenomena, and because of the high solids to water ratio in columns, a very long time is then needed to reach steady state operation where biodegradation potential can best be evaluated.

In order to reduce the above problems and yet maintain small microcosms for the reasons noted above, a semicontinuous slurry microcosm system was developed and evaluated. The aquifer material for study came from Moffett Federal Airfield, Mountain View, CA (Moffett Field), where a pilot-scale field testing of aerobic cometabolic biodegradation of trichloroethylene (TCE) had been carried out over several years (*1*−*3*, *8*−*12*). Here, methane, phenol, and toluene have been evaluated as potential primary substrates for inducing aerobic TCE cometabolism. The extensive database that resulted provided field evaluation against which the results of the laboratory study could be compared. In this study, the slurry microcosms were evaluated by using phenol and toluene as primary substrates. In addition, aquifer material from a TCE-contaminated site at Edwards Air Force Base, CA (Edwards), was then evaluated similarly to determine the potential there for *in situ* biodegradation of TCE.

Materials and Methods

Microcosms. Moffett Field aquifer material from bore SU39-16 was used to evaluate the semicontinuous slurry microcosm method and also to serve as a positive control for microcosm studies of Edwards aquifer material. Edwards aquifer material was obtained from bore SU42-1 at site 19, using cores of 15 cm length and 5 cm diameter from four different depths, 10.4, 12.2, 14.3, and 20 m. Using aseptic conditions, aquifer material from each core was equally distributed between eight sterile, 65 mL screw cap glass bottles (23 ± 5 g/bottle, dry weight), which were then filled without headspace using filter-sterilized oxygen-containing (1 mM) ground water (53 ± 5 mL/bottle). The microcosms were capped with Teflon-lined silicon septa and open-hole screw caps.

Incubation of Microcosms. Microcosms were incubated inverted in the dark at 20 °C on a rotating shaker (Lab Line Instruments, Mellrose Park, IL) at 180 rpm. Fluid and chemicals were periodically replenished as follows. Microcosms were centrifuged at 100 rpm for 15 min, 40 mL of clear supernatant was removed and replaced with oxygen-containing groundwater, respiked with primary substrate and TCE, and reincubated as described above. Both phenol and toluene were used as primary substrates. With each replenishment, 300 μL of a 19 mM phenol stock solution or 700 μL of a 6 mM toluene stock solution was added to the respective microcosms. In order to avoid possible substrate toxicity, both spikes were added in three separate pulses within the

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first 12 h of an incubation period, i.e., 3 × 100 μL for phenol and 1 × 300 μL plus 2 × 200 μL for toluene. All microcosms and controls were spiked at each replenishment with 20 μL of a 1 mM TCE stock water solution. These additions in effect resulted in a concentration in the 40 mL of replenishment feed of 32 mg/L dissolved oxygen (DO), 13.4 mg/L (0.143 mM) phenol or 9.7 mg/L (0.105 mM) toluene, and 660 μg/L (5.0 μM) TCE. The pH of the microcosms remained constant (7.2) without adjustment. The semicontinuous liquid exchange and reincubation noted above was repeated many times until steady state conditions were obtained.

Microcosm Sampling. Microcosms were sampled for DO, primary substrate, and TCE analyses before and after each incubation period. Since the culture DO concentration was above air saturation, and TCE and toluene are volatile, a special procedure was used to spike and sample the microcosms. The open-hole screw caps were removed from microcosms without displacing the septa. The amount of sample needed was pulled from the bottles by inserting the needle of a precision syringe between the septum and the rim of the bottles into the culture liquid. Volumes removed were carefully replaced immediately after sampling with sterile groundwater in order to prevent formation of a head space. Toluene, phenol, and TCE were added the same way, except that prior to their addition, the liquid volume of the microcosms was reduced by the volume of the primary substrate spikes to be added in order to prevent overflowing.

Analytical Methods. *Dissolved oxygen* (DO) was measured by a DO probe (Hansatech Instruments, Kings Lynn, England).

TCE was measured using a HP 5890 Series II gas chromatograph with a J&W Scientific DB1, 30 m, 0.53 mm i.d. megaboreR capillary column, and electron capture detector. Zero-grade helium carrier gas flow rate was 23 mL/min at a head pressure of 50 kPa. The makeup gas was Argon/Methane, 95/5% (V/V), at a flow rate of 67 mL/min. The injector and detector temperatures were 200 and 265 °C, respectively. Samples were analyzed at 60 °C with 3 min run time. TCE from 1 mL of standards and samples was extracted into 0.5 mL of HPLC-grade pentane, using 2 mL vials with Teflon-lined silicon septa. The vials were shaken for 30 min on a rotary shaker at 360 rpm. Then, 6 μL of the pentane phase was injected for analysis. The detection limit was 0.5 μg/L. Raw data were processed by a Perkin-Elmer Nelson System 900 series interface and a Perkin-Elmer Nelson 2600 Chromatograph Data System, respectively.

Phenol was determined by reverse phase HPLC on a Perkin-Elmer Series 400 liquid chromatograph, using a 250 mm Adsorbosphere C18 2 μm column (Alltech) and a 50 μL injection loop. The eluent consisted of 60% HPLC-grade methanol and 40% acetate buffer (50 mM, pH 4.5, sodium acetate and glacial acetic acid, analytical reagent grade). The flow rate was 1 mL/min at 3300 psi. Phenol was detected at 273 nm with a Hewlett-Packard Series 1050 spectrophotometer with flow-through cell. The detection limit was 2 μM. Samples, 0.5 mL, were transferred into 4 mL amber vials containing 20 μL of phosphoric acid (100 mM) and frozen at −20 °C. For analysis, frozen samples were thawed and centrifuged for 5 min at 1500*g* in a Clay-Adams Safety-Head centrifuge. Data were collected by a Perkin-Elmer Nelson System.

Toluene was measured using a HP 5890 Series II gas chromatograph fitted with a Quadrex 5022 capillary column, type 007-502, heated at 100 °C, and a photo ionization detector (200 °C). Zero-grade helium gas flow rate was 20 mL/min at 20 psi. A purge and trap injection system (Tekmar Model ALS sampler connected to a Tekmar Model 4000 dynamic head space concentrator fitted with an Alltech trap 9081) was used. Samples (40 μL) were added to sampling tubes containing 5 mL Milli-Q water and purged for 7 min at 30 °C. Desorption preheat time was 0.5 min at 110 °C and desorption time was 2 min at 150 °C. The trap lines and valves were kept constant at 100 °C and the injection port was 200 °C. The detection limit was 0.5 μM. Samples were stored in 200 μL of capped vials containing 10 μL of phosphoric acid (100 mM).

Toluene Oxygenase Evaluation. Limited analyses were conducted in order to evaluate the dominate oxygenase present in the microcosms. Toluene oxygenase gene probes were tested against DNA extracted from the microcosms. In addition, expected initial toluene oxidation intermediates were evaluated for confirmation.

The same toluene oxygenase probes and positive control strain DNA were used as previously applied to Moffett test field DNA samples (*4*): Methyl-monooxygenase probe on plasmid pG5H2836 from *Pseudomonas putida* PaW1, toluene dioxygenase probe on plasmid pDTG601 from *P. putida* F1, toluene *p*-monooxygenase probe on plasmid pMY421 from *P. mendocina* KR, toluene *m*-monooxygenase probe on plasmid pAB141Ava I from *P. pickettii* PK01, and toluene *o*-monooxygenase probe on plasmid pMS80 from *Burkholderia cepacia*. G4 Probes were labeled with dioxygenin-11-dUTP using the random primed method and the Genius DNA labeling and detection kit from Boehringer Mannheim.

DNA was extracted from 30 mL of microcosm samples using a method developed specifically for soil samples (*13*). Of each DNA sample, 10 μL was run on a 0.8% agarose gel for evaluation of the extraction procedure. Extracted DNA samples were filtered on a nylon membrane using a dot blot apparatus (Bio-Rad Laboratories, Hercules, CA), as described by the manufacturer. Toluene oxygenase probes were sequentially hybridized with blotted microcosm DNA using the Genius System for filter hybridization and the Lumi-Phos 530 chemiluminescence procedure for probe detection from Boehringer Mannheim. Between hybridizations, probes were stripped from the membrane according to the suppliers manual.

Evaluation of Cresol Formation. Glass vials (10 mL) were filled with air-saturated toluene- and phenol-fed microcosm samples and spiked with 1 mL of a toluene-saturated water solution, each, to result in an initial toluene concentration of 60 mg/L. Vials were sealed and incubated at room temperature. The toluene/dissolved oxygen ratio was such that only partial toluene oxidation could result, thus leading to a buildup of toluene oxidation intermediates. Samples were tested for cresol formation after two weeks. Cresol isomers were analyzed similarly as for phenol.

Evaluation of Active and Total Biomass. The Most Probable Number (MPN) technique was used for the quantification of the active microbial population and a total protein determination assay was used for total biomass estimates.

MPN. MPN was determined by 10× dilution series of microcosm samples using 96 microwell plates and color indication for cell positive wells (*14*). From each of the Edwards triplicate microcosm sets, 0.67 mL of each phenol- and toluene-fed microcosm triplicate, respectively, was combined with 18 mL of sterile mineral salts solution resulting in one 10^{-1} dilution. The microcosms were vortexed for 1 min prior to removing the sample. From each of the 10^{-1} solutions, a dilution series ranging from 10^{-2} to 10^{-12} was prepared using tubes containing 1.8 mL sterile LB-medium (*15*). Each dilution was distributed into eight wells of the microwell plate, 0.2 mL/well. Prior to sample addition, each well was amended with 20 μL of a 0.2% solution of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), which served as a color indicator for growth. The plates were incubated at 20 °C in the dark for 3 weeks. MPNs and 95% confidence intervals were determined by a computer program (*16*).

Protein Determination. Of an undiluted mixed microcosm sample, 1 mL was amended with 0.1 mL of a 10% solution of sodium dodecylsulfate (SDS) in 2 mL glass vials with screw caps. The samples were incubated in a boiling water bath for 15 min, and 0.6 mL was transferred into 10 mL glass tubes

containing 0.8 mL of Pierce micro bicinchoninic acid (BCA) protein assay working reagent (Pierce, Rockford, IL). After mixing, tubes were covered with aluminum foil and incubated at 60 °C for 1 h for color development and centrifuged at 1000 rpm for 5 min. The supernatant absorbency was measured at 594 nm and compared to that of bovine serum albumin protein standard samples (0, 10, and 20 mg/L) treated like microcosm samples.

Biomass Estimates from Protein. Biomass formed from phenol and toluene additions was used to determine biomass to protein ratios. One milliliter samples from all phenol- and toluene-fed microcosms, respectively, were combined and added to 250 mL bottles with mininert valves containing 150 mL of mineral salts solution (*17*), but no aquifer material. Cultures were incubated at 20 °C and 180 rpm shaking with the same concentrations of phenol and toluene, respectively, and with several fluid exchanges as for microcosms. For ash-free dry weight, four replicates of 225 mL aliquots from each phenol-fed and toluene-fed mixed culture were filtered through preweighed glass fiber filters, washed, dried at 100 °C for 3 h, and weight loss determined from combustion at 500 °C for 30 min. From the same cultures protein concentrations were determined. Toluene-fed mixed cultures contained 23 ± 1 mg/L dry cell weight and 9 ± 1 mg/L protein (40% dry cell weight). Corresponding phenol-fed mixed cultures were 27 ± 3 mg/L and 15 ± 2 mg/L, respectively, resulting in a cellular protein content of 54%.

Samples from the enrichment were also combined with aquifer material at the same solids/liquid ratio (1:4) as in microcosms. Measured protein concentrations here were 12 ± 1 mg/L without Edwards aquifer material and 6.2 ± 0.5 mg/L with aquifer material, indicating aquifer material caused a 48% diminution of the detachable protein content. This factor was taken into account for protein determination in microcosms. Moffett aquifer material, however, contained too much protein (10 ± 0.1 mg/L) to use this procedure for reliable estimates of biomass production from primary substrate consumption.

Results

Method Development. In order to develop the semicontinuous slurry microcosm system, the physiological requirements of the microbial population in Moffett aquifer material and groundwater were evaluated, including oxygen availability, substrate addition and concentration and nutrient sufficiency.

The effect of DO on phenol consumption rate was studied with duplicate microcosms amended with groundwater containing 0.12 mM phenol and four different initial DO concentrations (0.3, 0.6, 0.8, and 1 mM). From the following equation for complete phenol oxidation, 7 mol of DO are required per mole of phenol oxidized:

Phenol

$$C_6H_6O + 7O_2 = 6CO_2 + 3H_2O \qquad (1)$$

However, because some substrate is diverted for organism synthesis, DO requirement for phenol was found to be 0.59 $\pm$ 0.04 mM or 4.9 mol DO per mol of phenol. The oxygen demand of control microcosms not containing phenol (0.21 ± 0.05 mM) was first subtracted for this evaluation. Higher DO concentrations had no inhibiting effect on phenol degradation. However, when DO dropped below air-saturation (0.29 mM), phenol degradation rate decreased. In order to keep DO concentrations sufficiently high, initial DO concentrations of 1 mM were chosen for subsequent experiments.

In order to evaluate the effect of phenol concentration, duplicate microcosms with oxygen-saturated groundwater (1 mM) were incubated at four different initial phenol concentrations (0.06, 0.10, 0.17, and 0.22 mM). Oxygen-saturated conditions were insufficient for oxidation of 0.22 mM phenol; thus, 0.17 mM phenol was the highest concentration used in subsequent studies.

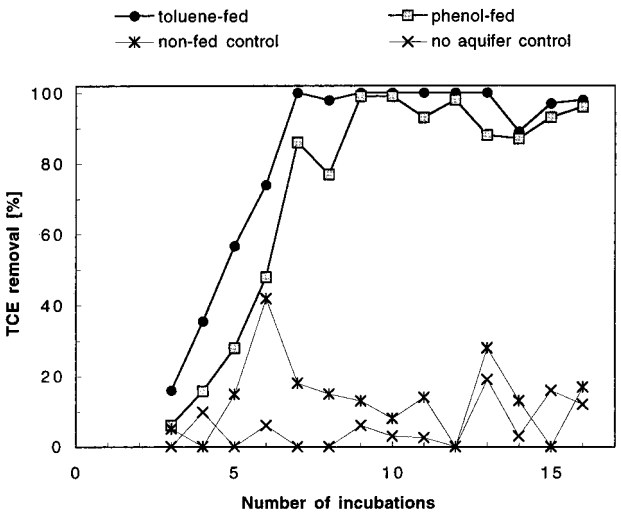

FIGURE 1. TCE removal as a function of incubation number in toluene- and phenol-fed Moffett microcosms. Loss of TCE due to sorption or leaking is represented by nonfed controls and no aquifer controls, respectively.

In order to evaluate nutrient sufficiency in the aquifer material and groundwater, nitrate, phosphate, $MgSO_4$, and a mixture of trace elements (Fe^{2+}, Mn^{2+}, Mo^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}) were added to separate microcosms, respectively. These supplements did not increase TCE removal nor DO consumption rates compared to nonamended microcosms. The bicarbonate in the groundwater provided an adequate buffer (pH 7.2). Thus, with the Moffett field system, no nutrient or buffer addition was necessary.

One critical factor was the way substrate was added to the microcosms, i.e., in three subsequent pulses over 12 h of an incubation period, rather than in one single spike. When the total mass of phenol was added in a single spike, TCE removal declined from 90 to 40% in subsequent incubations. Similar TCE removal decrease was observed for microcosms over four incubation periods with phenol only prior to TCE addition. TCE removal was high initially, but decreased to about 40% removal. Part of the initial 90% TCE removal might have been due to sorption rather than biodegradation. However, when phenol was added in three pulses, TCE removal remained at the 90% level. Thus, while phenol addition in one spike did not have an inhibitory effect with respect to phenol degradation or DO consumption, it did have a significant impact on TCE removal for some undefined reason.

TCE Removal in Moffett Microcosms. Using the optimized operating conditions described under Materials and Methods, Moffett microcosms were used to compare TCE removal efficiency at steady state with TCE removals obtained in the field (*1*−*3*). A set of triplicate Moffett microcosms was fed phenol, and for comparison, a second triplicate set was fed toluene. Duplicate microcosms were not fed primary substrate and served as controls for measurement of sorption of TCE and aquifer material oxygen demand. Two additional control microcosms contained no aquifer material and were exchanged with groundwater containing only TCE and DO to evaluate potential TCE and oxygen losses through leakage. TCE, toluene, phenol, and DO were measured at the beginning and end of each incubation period. Results were obtained over 16 subsequent incubations. The incubation periods were 15 days for the first incubation, 4 days for the second incubation, 2 days for the third to fifth incubations, and alternating 3 and 4 days of incubations thereafter (Figure 1).

TABLE 1. Summary of TCE Percentage Removals from Moffett Microcosms[a]

	TCE in feed (μg/L)	primary substrate in feed (mg/L)		TCE removal (%)		oxygen/primary substrate (mol/mol)	
		toluene	phenol	toluene	phenol	toluene	phenol
microcosms	660	9.7	13.4	94 ± 1	94 ± 3	5.9 ± 0.1	4.9 ± 0.2
in situ[b]	230	9.0	12.5	93	94	6.0	4.0–5.6

[a] Results are averages and standard deviations for triplicates and eight successive incubations of triplicates during steady state operation. [b] Hopkins and McCarty (*3*).

No TCE was added in the first two incubations. TCE removal increased with each subsequent incubation, and reached a steady state of 98 ± 1% and 94 ± 3% for toluene- and phenol-fed microcosms, respectively, after the ninth incubation (Table 1). Loss of TCE due to sorption in non-fed controls was 16 ± 8%, TCE leakage in controls containing groundwater was 8 ± 4%. However, both losses can be expected to be significantly lower in regular microcosms, both because biological removal of TCE lowered the sorption pressure and aquifer material served as additional seal against escape in invertedly incubated microcosm bottles. Thus, no correction for such losses was made. The amount of DO used under steady state conditions was 5.9 mol/mol and 4.9 mol/mol for toluene and phenol, respectively. These values represent 67 and 70%, respectively, of the theoretical maximum oxygen consumption for complete oxidation. The nonoxidized portion probably was incorporated into cell mass.

TCE removal efficiency and oxygen consumption in Moffett microcosms at steady state (Table 1) were similar to that found *in situ* at the Moffett field test site (*2*, *3*), except that the TCE removal efficiency with toluene was somewhat higher in the microcosms. The DO usage at Moffett Field with phenol feed increased over time due to respiration from an increasing bacterial population. The phenol-microcosm DO consumption was intermediate between the highest and lowest field consumption values. The field DO consumption for the toluene case did not vary in a similar fashion as toluene was added after a steady state population had already been established. Overall, the microcosm results were similar, or perhaps in the case of toluene, somewhat better than the field results.

TCE Removal in Edwards Microcosms. The same microcosm approach used for Moffett aquifer material was used to evaluate the potential for TCE cometabolic degradation in aquifer material and groundwater from Edwards Air Force Base. Eight microcosms were prepared with aquifer material from each of four depths for a total of 32 microcosms. As with Moffett aquifer material, triplicate microcosms from each depth were fed phenol, a second triplicate set received toluene, duplicate microcosms were not fed a primary substrate, and two control microcosms contained no aquifer material and were exchanged with groundwater containing only TCE and DO.

Incubation periods and number were the same as for Moffett microcosms. During the first incubation period of 15 days, microcosms were fed either phenol or toluene. The extent of primary substrate degradation gradually increased until the ninth incubation period, when substrates were consumed below their detection limits. Averaged results for all microcosms are illustrated in Figure 2. TCE removal increased with each successive incubation, reaching maximum values only after primary substrates were completely degraded following the ninth incubation.

TCE removal in the toluene-fed 20 m microcosms approached 100 ± 0% removal after seven incubation periods (Figure 3a), but in the 14.3 m microcosms reached only 87 ± 5% removal by the 15th incubation period (Figure 3b). Differences between phenol-fed microcosms were similar. Reasons for the large fluctuations between depths are not

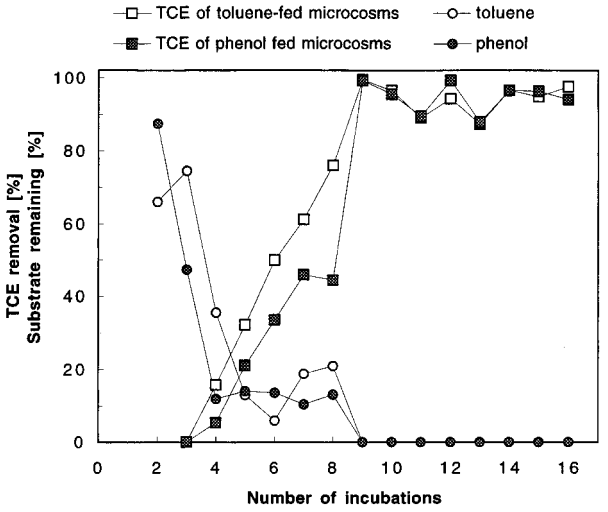

FIGURE 2. Change in primary substrate degradation and TCE removal with incubation number for Edwards microcosms. Data points represent averages of results for all microcosms at all depths.

clear, but may reflect differences in TCE removal ability of dominant organisms at each depth. The results for the last eight incubations for each set of triplicates (steady state conditions) were averaged (Table 2). Differences between toluene- and phenol-fed Edwards microcosms from a given depth were small. Differences between depths, however, appeared significant.

Estimation of Biomass. An estimation of active and total biomass in Edwards microcosms was first attempted using MPN and protein measurements. Samples from two sequential incubations after 30 incubation periods were obtained from all Edwards microcosms and used for these analyses. MPN values varied considerably from one microcosm to the next. In toluene-fed microcosms MPNs ranged from 10^4 to 10^7 cells/mL with a median of 3×10^6 cells/mL. Phenol-fed microcosms were similar with MPNs ranging from 10^5 to 10^7 cells/mL and a median of 10^6 cells/mL.

Biomass measurements from protein analyses were less variable. Biomass concentrations so estimated, referenced to the 53 mL pore volume, ranged between 22 and 42 mg/L with an average of 33 mg/L and standard deviation of 7 mg/L for toluene fed microcosms, and between 28 and 52 mg/L with an average of 42 mg/L and standard deviation of 9 mg/L for phenol-fed microcosms (Table 3).

An indirect method was then used for estimating total biomass from oxygen to primary substrate consumption ratios. Here, the difference between theoretical oxygen consumption for complete substrate oxidation and the measured value represents the portion of primary substrate converted to biomass or to biomolecules, either soluble or particulate. Estimates of microcosm biomass made on this basis are likely to be somewhat high because of losses in both soluble and particulate biomolecules with each fluid exchange. Nevertheless, this approach should provide an upper bound on the biomass estimates. First, an overall equation was written for primary substrate oxidation and biosynthesis reactions, assuming that an empirical cell formula of $C_5H_7O_2N$

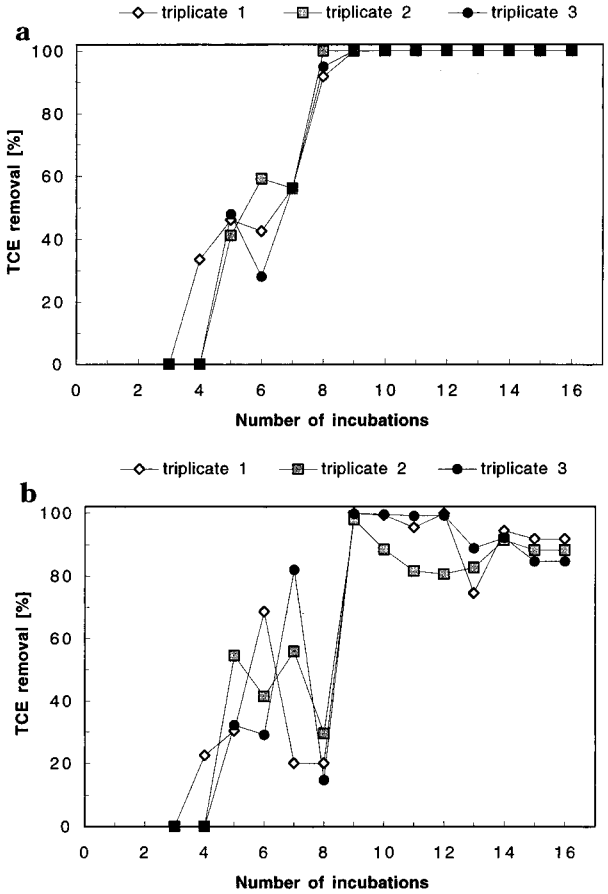

FIGURE 3. Comparison of TCE removal between triplicates for toluene-fed Edwards microcosms (a) 20 m aquifer material and (b) 14.3 m aquifer material. Phenol fed microcosms behaved similarly.

TABLE 2. Comparison of TCE Removal between Toluene- and Phenol-Fed Edwards Microcosms for Different Depths[a]

	TCE % removed	
sample depth (m)	toluene-fed	phenol-fed
10.4	99 ± 2	98 ± 2
12.2	87 ± 17	94 ± 4
14.3	90 ± 6	91 ± 5
20.0	100 ± 0	99 ± 2
average and population standard deviation	94 ± 6	95 ± 2

[a] Results are averages and standard deviations of triplicates over eight successive incubations during the steady state TCE-removal phase.

(*18*) applies:

toluene oxidation

$$C_6H_5CH_3 + 9f_eO_2 + \frac{9f_s}{7}(H^+ + NO_3^-) \rightarrow \frac{9f_s}{7}C_5H_7O_2N + \left(7 - \frac{45f_s}{7}\right)CO_2 + \left(18f_e + \frac{99f_s}{7} - 14\right)H_2O \quad (2)$$

phenol oxidation

$$C_6H_5OH + 7f_eO_2 + f_s(H^+ + NO_3^-) \rightarrow f_sC_5H_7O_2N + (6 - 5f_s)CO_2 + (14f_e + 11f_s - 11)H_2O \quad (3)$$

where, f_e represents the fraction of primary substrate oxidized and f_s is the fraction synthesized and equals $1 - f_e$. Oxygen consumption initially was 4.6 ± 1 and 3.6 ± 1 mol/mol of primary substrate consumed for toluene and phenol, respectively. Since 9 and 7 mol of oxygen are required for their respective complete oxidation, f_e here equals 0.51 for both toluene and phenol. During steady state operation with higher biomass concentration, oxygen consumption increased to 6.0 ± 0.1 and 4.9 ± 0.3 mol/mol, resulting in higher f_e values of 0.67 and 0.7 for toluene and phenol, respectively. Using the above ratios and equations for mass balance, calculated biomass net yield is 0.77 and 0.59 g of biomass/g of toluene or phenol, respectively, during the start-up phase, and 0.52 and 0.40 g of biomass/g of toluene or phenol consumed, respectively, during steady state operation. Over the 30 fluid and substrate exchanges in the microcosms being considered, 10.7 mg of toluene and 14.8 mg of phenol were consumed per bottle. Using the steady state net yield values and a bottle pore volume of 53 mL, the calculated biomass concentration, if none were lost from the microcosms, would be 105 and 112 mg/L, for the toluene- and phenol-fed microcosms, respectively (Table 3). The protein-based total biomass estimates are 31 and 37%, respectively, of these maximum estimates. Since some biomass was undoubtedly lost with each exchange, the protein-based measurements for total biomass appear to be reasonably consistent with the oxygen-consumption-based estimates.

Both protein-based and oxygen-consumption-based estimates provide information on total biomass rather than on active phenol or toluene consuming biomass. An approach to estimating steady state active biomass resulting from organism growth and decay, makes use of the following growth and decay equation:

$$\frac{dX_a}{dt} = Y\left(-\frac{dS}{dt}\right) - bX_a \quad (4)$$

where X_a (mg/L) is the active organism concentration, Y (mg/mg) is the bacterial yield from primary substrate (phenol or toluene) utilization, $-dS/dt$ (mg L^{-1} day^{-1}) represents rate of primary substrate utilization, and b (day^{-1}) represents organism decay rate. After numerous exchanges, and assuming no loss of microorganisms in the effluent, X_a should reach a steady state value [$X_{a(ss)}$, representing a balance between growth and decay]. From a mass balance around the microcosm and assuming primary substrate is essentially 100 percent consumed within the microcosm (a valid assumption here), this steady state concentration is given by the following:

$$X_{a(ss)} = \frac{YS^\circ Q}{bV_p} \quad (5)$$

where Q represents the rate of fluid exchange (40 mL each of 3.5 day or 11.4 mL/day), V_p is the pore volume of the microcosm (53 mL), and S° represents the feed concentration of primary substrate (9.7 and 13.4 mg/L, respectively, for toluene and phenol). The yield value Y might be taken from the biomass yield estimates from oxygen consumption during microcosm start-up, which were 0.77 and 0.59 g of biomass/g of substrate for toluene and phenol, respectively. No information is available here for the decay coefficient, but typically it lies between 0.1 and 0.2/day (*19*). For example, Shurtliff *et al.* (*20*) reported a value of 0.12/day for phenol. Using a value of 0.15/day for b, $X_{a(ss)}$ is estimated to be 11 mg/L for both toluene and phenol enrichments (Table 3). The $X_{a(ss)}$ active population estimates represent 33 and 26% of the protein-based total biomass measurements for toluene- and phenol-fed microcosms, respectively, values that would appear reasonable.

Primary Substrate and TCE Biodegradation Rates. TCE removal and toluene- and phenol-degradation rates were evaluated twice during steady state TCE removal, the results of one such study are illustrated in Figure 4. As expected from competitive interactions between TCE and the primary

TABLE 3. Summary of Measured and Calculated Numbers for Active and Total Biomass, Growth Yields, and Estimated TCE and Substrate Degradation Rate Constants for Edwards Microcosms

			toluene-fed	phenol-fed
biomass (mg/L)	active	calculated ($X_{a(ss)}$)	11	11
	total	measured (protein)	33	42
		calculated (DO change)	105	112
yield (mg/mg)	start-up (Y)		0.77	0.59
	steady state (Y_{net})		0.52	0.40
TCE degradation rate (L/mg/day)	k'		0.07−0.15	0.08−0.13
primary substrate degradation rate (mg/mg/day)	k_s		1.5	3

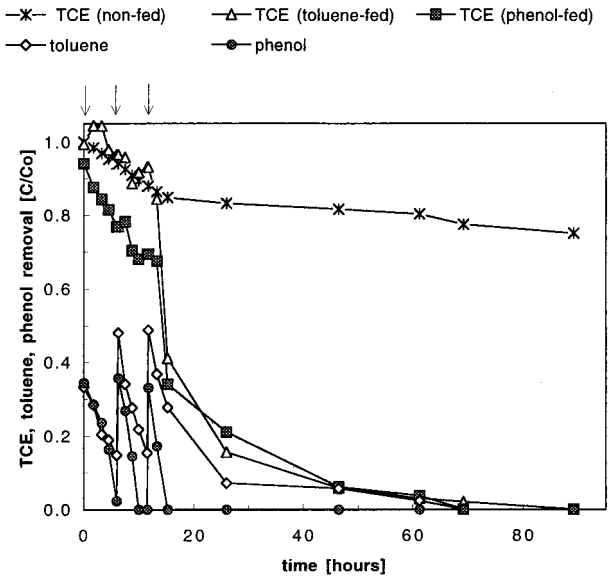

FIGURE 4. TCE and primary substrate removal during an incubation period under steady state conditions. Data points represent averages of results from two incubations for toluene- and phenol-fed Edwards microcosms. Arrows represent substrate pulses.

substrates (*21*−*23*), an incubation period was marked by an initial phase of almost exclusive degradation of primary substrate followed by a phase of significant TCE removal. Phenol- and toluene-fed microcosms differed somewhat, toluene removal being slower and not complete between spikes. TCE removal did not begin in the toluene system until after the third spike was added. In spite of these differences, there was little significant difference in the net TCE removals between the toluene and phenol systems (Table 2). This was the case in the Moffett field study as well (Table 1).

Rate coefficients for primary substrate and TCE degradation are useful for mathematical modeling and predictions of field results. Since competitive inhibition was quite significant, this should be considered in model development, but the information obtained from the microcosms was inadequate for this purpose. However, the results of primary substrate and TCE degradation can be used to estimate an overall rate coefficient that would be somewhat low because of competitive effects. For this purpose, the following rate equations were used:

$$-\frac{dS}{dt} = \frac{k_s S}{K_s + S} X_a \tag{6}$$

$$-\frac{dC}{dt} = \frac{k_c C}{K_{sc} + C} X_a \approx \frac{k_c C}{K_{sc}} X_a = k' C X_a, \quad \text{when } C \ll K_{sc} \tag{7}$$

where, S (mg/L) and C (mg/L) are the primary substrate and contaminant (TCE) concentrations, k_s (day^{-1}) and k_c (day^{-1}) represent the maximum rate of primary substrate and contaminant consumption per unit of active biomass per day, and K_s (mg/L) and K_{sc} (mg/L) are the affinity constants for the primary substrate and contaminant, respectively. Also, for the contaminant, when C is much less than K_{sc}, a reasonable assumption here, then the approximation shown on the right side of eq 7 is appropriate. Equation 7 can be integrated for TCE removal over an incubation period to give

$$C_e = C_i e^{-k' X_a t} \tag{8}$$

where C_e (mg/L) is the effluent concentration after an incubation period and C_i (mg/L) is some initial concentration. The value t is the reaction time, which here might be considered the time period over which TCE degradation occurred (typically 3.5 day minus 0.75 day of lag time or 2.75 day). C_i can be determined from a mass balance on the microcosm at the time of an exchange:

$$C_i = \frac{C_o V_e + C_e (V_p - V_e)}{V_p} \tag{9}$$

Here, V_e represents the volume of fluid exchanged or 40 mL. With eqs 8 and 9, and considering the efficiencies of removal listed in Table 2 for the various microcosms, the values for k' listed in Table 3 were obtained assuming an active biomass concentration of 11 mg/L. The results suggest that calculated percentage removals for the microcosms are very sensitive to values of k'. The differences in k' values between toluene and phenol enrichments do not appear significant compared with TCE removal differences found with sample depth. Indeed, k' varied by a factor of just over 2 for all active biomass results or all total biomass results.

Using the active biomass concentration of 11 mg/L and the rate of primary substrate utilization illustrated in Figure 4, the maximum primary substrate utilization rate constant k_s from eq 6 was also estimated, with results as listed in Table 3.

Oxygenase Evaluation. Mixed 10 mL samples from each of the different Moffett and Edwards triplicate microcosms were combined, resulting in one 30 mL sample for each aquifer sample and primary substrate. The procedure was repeated twice. Positive signals from hybridization for toluene *o*-monooxygenase (Tom) were found for all samples (Table 4). Positive signals for other toluene oxygenases were less frequent. Moffett field microcosms also indicated a dominance of toluene o-monooxygenase (*4*; Table 4). The dominant expression of Tom was confirmed by the detection of *o*-cresol in microcosms to which toluene was added in excess of oxygen availability. *o*-Cresol is the first toluene degradation product produced by toluene *o*-monooxygenase (*24*). *o*-Cresol formation from toluene was also found in Moffett field studies (*3*). Neither *m*- nor *p*-cresol, products from meta and para toluene cleavage pathways (*25, 26*), were detected in the field or in the microcosms incubated with toluene. No analyses were conducted for the presence of toluene dihydrodiol or benzylalcohol, the products from toluene degradation via toluene dioxygenase (*27*) or methyl-monooxygenase (*28*), respectively. Upon the basis of the enzyme probes and o-cresol formation, Tom appears to be

TABLE 4. Hybridization of Edwards and Moffett Microcosm DNA with five Toluene Oxygenase Probes and Comparison with Results from in Situ DNA Samples[a]

		toluene oxygenase				
substrate	microcosm	ortho-MO[b]	meta-MO[b]	para-MO[b]	methyl-MO[b]	TDO[c]
toluene	Edwards 10.4 m	+	(+)			(+)
	Edwards 12.2 m	+				
	Edwards 14.3 m	+	(+)			(+)
	Edwards 20.0 m	+	(+)			
	Moffett	+	(+)	(+)	(+)	
phenol	Edwards 10.4 m	+			(+)	
	Edwards 12.2 m	+			(+)	
	Edwards 14.3 m	+			(+)	
	Edwards 20.0 m	+	+	+	(+)	(+)
	Moffett	+	(+)		(+)	
	in-situ Moffett[d]	+				

[a] Experiments were carried out twice with newly extracted DNA. Results in brackets indicate where positive results were obtained from one experiment, only. [b] MO = monooxygenase. [c] TDO = dioxygenase. [d] Fries *et al.* (*4*).

the dominant enzyme acting on toluene, phenol, and TCE at both the Moffett and Edwards sites.

Discussion

Aerobic TCE biodegradation was successfully stimulated in the semicontinuous batch microcosms by addition of either toluene or phenol as primary substrates. TCE and primary substrate removal efficiencies, oxygen to primary substrate ratios, and oxygenase enzyme expression were all reasonably similar between slurry microcosms and Moffett Field *in situ* field results. Laboratory microcosm and Moffett groundwater has similar temperatures (20 and 18 °C, respectively). The only difference was that the microcosms were somewhat more efficient at TCE removal with toluene as the primary substrate.

The semicontinuous batch microcosm method was also used to predict performance of *in situ* cometabolic biotransformation of TCE using aquifer material from the Edwards Air Force Base site where a full-scale evaluation of the process was proposed. Samples from several depths and one location that spanned two different aquifers were evaluated. The results were very similar to those obtained with Moffett aquifer material. The average percentage of TCE removed was similar using similar primary substrate concentrations. Oxygen demand was essentially identical, and the dominant oxygenase appeared to be Tom, as was the case at Moffett Field.

There was, however, some noticeable difference in the performance with Edwards aquifer material taken from different depths. The shallowest sample (10.4 m) and deepest sample (20.0 m) approached steady state removal faster and provided higher TCE removals (98−100%) with both phenol and toluene than samples from the intermediate depths (12.2 and 14.3 m), which provided TCE removals in the 87−94 percent range. These differences appear to be statistically significant as they resulted with either primary substrate and in the triplicates used. Reasons for such differences are not clear, but they likely result from differences in the bacteria that come into dominance in the respective locations. For example, Fries *et al.* (*4*) found that many different toluene and phenol degrading species were present in the Moffett aquifer, with widely differing abilities to degrade TCE. The actual TCE degrading capability of a given mixed population that happens to develop in one aquifer sample is thus likely to differ greatly from another. This is why microcosm studies are highly desirable before instigating aquifer remediation by cometabolism.

An important question is how does the Edwards microcosm studies mimic the full-scale Edwards field results? Results of steady state operation at Edwards are now available and indicate that with toluene injection of 13.6 mg/L, 86−87% TCE removal is being obtained. The toluene concentration is somewhat higher than the 9 mg/L toluene used in the microcosms, and the 86−87% TCE removal is somewhat lower than the average of 95% removal obtained in the microcosms. However, the field results indicated are for the upper aquifer and are similar to those obtained with the 12.2 m aquifer material (87%). In a similar fashion, the toluene-fed Moffett microcosms gave higher TCE removals than obtained in the field. These results indicate that samples from several depths should be evaluated when field applications are being considered, and that one should use a somewhat conservative approach in using microcosm results for estimating field results.

The microcosm method used here is similar to that used by many others for similar purposes. Some comments about findings with respect to their operation are appropriate. Preliminary studies revealed that oxygen saturation did not inhibit TCE removal. Such tolerance for high DO concentrations is important as aquifer DO demand, as well as that for primary substrate oxidation, can significantly deplete the DO in the microcosms. Without DO present, TCE degradation will not occur. The DO demand of Moffett aquifer material alone, based upon nonfed controls, represented about 20% of the total oxygen demand with the slurry method used here, a problem that has been much more significant in column studies with the same material where the aquifer solids/water ratio was much higher (*5*, *7*). Such high DO demand in aquifer material appears to be characteristic of disturbed material as it has not been observed in Moffett Field studies (*9*). The lower aquifer material DO demand exhibited with the slurry microcosm represents a distinct advantage over the column microcosm.

The nutrient requirement of microcosm cultures was here met by nutrients present in groundwater and aquifer material. However, this might not always be the case and must be evaluated for each site of interest. The lack of sufficient nitrogen in soil has been shown to impair biodegradation of toluene (*29*) and phenol (*30*).

TCE removal efficiency in the slurry microcosms was similar to or perhaps slightly higher than that obtained with column microcosms (*7*) or at Moffett Field (*3*). A major advantage of the low solids/liquid ratio in the slurry microcosms is that TCE sorption was reduced significantly, permitting steady state removals to be obtained quicker. Another significant advantage of the slurry microcosms is that for a given amount of aquifer material, liquid sample volume for analyses at each exchange was much greater than with the column approach. In addition, since slurry microcosms are readily mixed, the samples obtained contain a true composite average of the concentrations of materials of interest within the microcosms. Finally, the slurry microcosms can readily be sampled for total biomass concentration determinations, an analysis that is exceedingly difficult with column microcosms.

A disadvantage of the slurry approach includes the apparent necessity in this case to add the toluene or phenol in three successive pulses during the initial day of each exchange in order to obtain TCE removal similar to that in the field. This was not necessary with column microcosms. The cause of this problem with the slurry microcosms is not clear, but may be related to the toxicity of these primary substrates, even at low concentrations. Fries *et al.* (*4*) found many more phenol- and toluene-consuming TCE degraders could be isolated from Moffett sediments using 5 mg/L phenol and toluene rather than 50 mg/L for culture growth. Such a toxicity problem does not seem to exist significantly in the field or in column microcosms, perhaps because bacteria in micropores are not exposed to the higher concentrations.

The rates of primary substrate utilization, k_s, and that of TCE biodegradation in the slurry microcosms were estimated as the ratio of k/K_s, or k'. The values so obtained were based upon an estimate of active biomass concentration. The values

obtained (Table 3) might be compared with that of others. Semprini *et al.* (*9*) selected a value for k' of 0.03 L/mg/day based upon estimates of X_a as this allowed good model simulation of results from studies at Moffett Field. The comparable values from Table 3 for active organisms of 0.08–0.13 L/mg/day are considerably higher. Perhaps more definitive comparisons can be made with data from controlled chemostat studies. From a laboratory phenol-fed mixed culture chemostat study, Shurtliff *et al.* (*20*) reported a k' also of 0.03 L/mg/day. However, Chang and Alvarez-Cohen (*32*), using chemostat grown cultures at a 5 day detention time, reported a k' of 0.10 day^{-1} for phenol and 0.02 day^{-1} for toluene. Their phenol value is similar to the Table 3 values but their toluene value is much smaller.

In further comparisons of rate coefficients for primary substrate utilization, Shurtliff *et al.* (*20*) reported values of Y, k, and b for phenol of 0.61 mg/mg, 9.3 mg of phenol/mg of cells/day, and 0.12 day^{-1}, respectively. Their Y value is similar to that found here, but their k_s value is much higher. Chang and Alvarez-Cohen (*32*) had somewhat lower Y values than found in this study or 0.29 and 0.55 mg/mg for toluene and phenol, respectively. However, their values appear to be net rather than maximum yields since they did not correct for organism decay. Differences noted no doubt result partly from different approaches used to estimate X_a and to the use of somewhat different models. However, when one considers the widely varying TCE removal abilities of toluene and phenol using organisms (*4*), the results then appear surprisingly close. This analysis illustrates another advantage of the slurry approach as such an analysis of rate coefficients would be most difficult to do with column studies.

A question of importance for field operation is what factors under operator control have the most impact on the performance of TCE removal efficiency. This might be answered in part from a combination of eqs 5 and 7. For continuous introduction at a source well of TCE-contaminated groundwater containing primary substrate and oxygen, and with plug-flow from the well into the aquifer, integration yields:

$$\ln \frac{C_e}{C_i} = -\frac{k'YS^\circ}{b} \text{ or } \frac{C_e}{C_i} = e^{-k'YS^\circ/b} \qquad (10)$$

Here, C_i represents the TCE concentration in the water entering the aquifer and C_e is the concentration leaving the treatment zone. The time t resulting from integration is equal to the time it takes for the injected fluid to fill the bioactive zone V_p in the aquifer, which is given by the ratio V_p/Q. Thus, these terms cancel each other in the combined result given as eq 10. While these are obviously great simplifications, the resulting eq 10 can provide insights into the influence of some major variables. One important result is that time is not a factor in eq 10. This is one reason why the exchange time of 3.5 days in the laboratory slurry reactor does not need to equal the flow time through the bioactive zone at the Moffett test site, which was less than 2 days, to provide comparable results. Equation 10 also indicates that efficiency of removal is a direct function of primary substrate concentration in the feed stream, as demonstrated at the Moffett field site (*1*). This indicates why it is important in laboratory microcosm studies to maintain the primary substrate feed concentration the same as in the field if one is to simulate field results. Equation 9 suggests that the primary substrate concentration in the field should be maintained as high as possible to achieve maximum TCE removal efficiencies. However, there is a major limitation on the upper concentration that can successfully be used because of limitations in maintaining high DO concentrations. Another factor of importance is the yield value, Y, which is generally higher for phenol- or toluene-oxidizing than for methane- or ammonia-oxidizing bacteria. An operational factor of importance then is the product YS°, which should be as high as possible, as limited by the supply of oxygen, in order to achieve maximum TCE removal efficiency.

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